

Detection of Cystic Echinococcosis Antigen Concentration in Serum Patients

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Abstract

Cystic Echinococcosis (CE) or Hydatidosis, a parasitic disease that caused by larvae of *Echinococcus granulosus*, is an important zoonosis endemic in large parts of the world and Iraq. Diagnosis of hydatidosis antibodies is based on immunodiagnostic methods (ELISA), along with radiological and ultrasound examinations, when treatment by chemotherapy and surgery. The objectives of the present study were to determine a specific antigen concentration-based ELISA technique for diagnosis of hydatidosis and compare it before and after surgery. A total sample of 102 Iraqi subject from different hospitals were included in this study. They were distributed as 52 patients (43 females and 9 males, included the same group of patients study after 3-7 days surgery to remove hydatid cyst), and 50 persons healthy controls. Hyper immune-serum was raised against hydatid cyst fluid in rabbits. Anti-hydatid cyst IgG was purified by Ione exchange chromatography. The results show that increased circulating hydatid cyst antigen concentration before surgery 38.7 μ g/ml than antigen concentration after surgical removal hydatid cyst 25.7 μ g/ml the difference was significant at $P \leq 0.01$. In the present study, the presence of circulating antigens in sera of hydatidosis patients by indirect ELISA [new kit] was detected hydatid cyst antigen in preoperative sera in 50 out of 52 (96.15%), the test demonstrated antigen in 48 out of 52 (92.30%) postoperative sera.

Key words: hydatidosis, antigen, serum.

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الكشف عن تركيز مستضد داء المشوكات الكيسي في مصل المرضى

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الخلاصة

داء المشوكات الكيسي او داء الاكياس العدرية، مرض طفيلي يتسبب عن الطور اليرقي للمشوكة الحبيبية، وهو من الامراض المهمة المشتركة بين الانسان والحيوان، يتوطن في اجزاء كبيرة من العالم والعراق. تشخيص الاجسام المضادة لداء المشوكات يعتمد على طرائق التشخيص المناعية (الاليزا) مع الفحوصات الشعاعية والموجات فوق الصوتية (السونار)، بينما يعالج بالعلاج الكيميائي والجراحة. الهدف من هذه الدراسة لتحديد تراكيز المستضدات الخاصة بالاعتماد على تقنية الاليزا لتشخيص داء المشوكات والمقارنة قبل وبعد الجراحة، تضمنت الدراسة مامجموعه 102 عينة عراقية من مختلف المستشفيات، موزعة 52 مريض (43 اناث و 9 ذكور قبل الجراحة، نفس المجموعة السابقة درست بعد 3-7 أيام من ازالة الاكياس العدرية جراحيا)، و 50 شخصا من الاصحاء كمجموعة سيطرة . منعت الارانب ضد سائل الكيس العدري، نقيت الاجسام المضادة للاكياس العدرية IgG الناتجة من التمنيع من خلال كروماتوغرافيا التبادل الايوني، بينت النتائج زيادة تركيز المستضدات الدائرة في المصل قبل الجراحة 38.7 مايكروغرام / مل مقارنة بتركيزها بعد ازالة الكيس 25.7 مايكروغرام / مل، ظهرت فروق معنوية تحت مستوى احتمالية اقل من 0.01، بينت الدراسة الحالية ظهور المستضدات الدائرة في مصل مرضى الاكياس العدرية من خلال الطريقة الغير مباشرة للاليزا (عدة جديدة مصنعة)، كشف عنه في مصل المرضى قبل الجراحة في 50 من اصل 52 (96.15%)، كشف الاختبار المستضد في 48 من اصل 52 (92.30%) لمصل المرضى بعد الجراحة.

الكلمات المفتاحية : داء المشوكات، المستضد، المصل.

Introduction

Cystic Echinococcosis (CE) is a silent cyclozoonotic infection of humans and domestic animals caused by larvae of the dog tapeworm *E. granulosus*. It has a worldwide distribution and variable geographic incidence [1]. A great number of immunological assays have been developed for detection of anti-hydatid cyst antibodies [2, 3, 4, 5], although recently molecular

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biology techniques have also been applied. The circulating antibodies persist in the circulation for a long time; even after removal of the cyst by surgery or a clinical cure by chemotherapy[3]. However, detection of serum antibody has a major drawback in that demonstration of specific antibodies against hydatid antigen cannot differentiate between recent and past infections [6]The detection of circulating antigens rather than antibodies might be very useful in the immunodiagnostic of hydatid disease [7,8]. After surgery or chemotherapy it is often difficult to be sure whether the patient is cured of hydatid disease or remains infected. This is a recognized problem in the management of hydatid disease, and is due to the persistence of small or ectopic cysts in other sites even after surgery, the possibility of secondary Echinococcosis following surgery of hydatid cysts, or the development of resistance of cysts to chemotherapy [9].The detection of circulating antigens rather than antibodies might be very useful in the immunodiagnostic of hydatid disease [7,8].The aim of the present study was to develop ELISA method(new kit), using hyper immune serum raised in rabbits against sheep hydatid cyst fluid antigens, to detect circulating antigen in both pre and post –operative sera.

Material and methods**Subjects:**

A total sample of 102 Iraqi subjects was enrolled in the study. They were distributed as 52 (43 females and 9 males, included the same group of patients before and 3-7 days after surgery to remove hydatid cyst) patients (age range: 5 –60 years), 50 controls (control 40 females and 10 males); age range: 15 – 50 years. The patients were referred to the surgery Units at Baghdad Teaching Hospital Medical city hospital /Baghdad and Diyala province / Baquba Hospital General, during the period from January 2014 – till April, 2015. The diagnosis was made by the consultant medical staff at the two hospitals, and it was based on a clinical examination, X-ray, CT scan, Ultrasound findings and laboratory tests.From each participating subject, a total of ten ml of venous blood was collected using disposable syringes The blood was transferred to two plain tubes, which were left in the refrigerator (4°C) until clotting for approximately 30 minutes, and by then they were centrifuged (3000 rpm) for 15 minutes in a temperature cooled centrifuge (4°C). After centrifugation, the serum was separated and distributed into aliquots (0.25 ml) in 0.5ml Eppendorf tubes, which were deep free zed immediately at -20°C until used.

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Hydatid cyst Antigen:

For the antigen preparation according [10], hydatid cysts were obtained from freshly slaughtered and heavily infected sheep at the local abattoirs. The daughter cysts were taken from intact hydatid cysts dissected from sheep liver HCF Ag was Cooling Centrifuge (Kokusan D-300H Japan) centrifuged at 18,000 rpm for 30 minutes at 4 °. The supernatant, the hydatid cyst fluid antigen (HCF Ag), was stored at -20 ° C in 1 ml aliquots, after determining the protein content by Brad ford, method [11].

Hyper immune hydatid antiserum preparation:

Hyper immune hydatid antiserum was raised in rabbits as per the procedure described by [12] with some modification. Two healthy, adult male, white New Zealand rabbits (3–4 kg, which were obtained from the National Center of Researches and Drugs Monitor in Baghdad), they were adapted at the animal house (of Education for Pure Science Ibn Al-Haitham college in Baghdad) for 2 weeks before starting experiment, (they were fed on commercial assorted pellets and clean water), five to six months of age were used for raising antisera against the hydatid antigen. The rabbits were immunized with 1.5 ml of hydatid antigen, having a protein concentration of 8 mg/ml, HCF was emulsified with an equal volume of Freund's complete adjuvant with 0.5 mL of this emulsion in all four limbs intramuscularly. After six weeks, they were re injected intramuscularly with 0.5 mL of the same antigen in Freund's incomplete adjuvant in each limb. After 10 days, blood samples were taken by ear vein bleeding, and the serum was checked for the presence of anti-hydatid antibodies, by Double diffusion test was performed as previously described [13], gel diffusion showed distinct bands of precipitation, When the antibody titer was significant (1:1024 and above) in test bleeding, subsequently blood was collected from rabbits for polyclonal antibodies.

Immunoglobulins precipitated:

Antiserum was purified as per the method described by Gottstein, [14]. Briefly, 1 mL of cold serum was mixed with 1 mL of cold saline, pH 7. The serum-saline mixture (2 mL) was added drop wise to 2 mL of cold saturated ammonium sulfate, pH 7, with stirring for 30 minutes on ice and then centrifuged at $3,000 \times g$ for 15 minutes at 0°C. The supernatant was then discarded

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and the precipitate was suspended in 2 mL of saline and the procedure was repeated until the supernatant was colorless. The final precipitate was suspended in 1 mL of saline and dialyzed against phosphate-buffered saline (PBS), pH 7.2, to remove all the residual ammonium sulfate. The titer of the purified antiserum was 1:2,048 by Double diffusion [13].

1- Separation and purification IgG by Ion Exchange chromatography:

Ione exchange chromatography was used to purify IgG from immunized Rabbit sera, by Diethylaminoethyl- Cellulose (DEAE-Cellulose) [15].

2-Estimation of concentration of IgG in the sample:

Used many from different standard (IgG) 0 , 10, 50 and 100 $\mu\text{g}/\text{ml}$ then draw the standard curve to estimation IgG test that diluents of sample (IgG) (A stock) (B 1/10) (1/20) was Estimation by the standard curve.

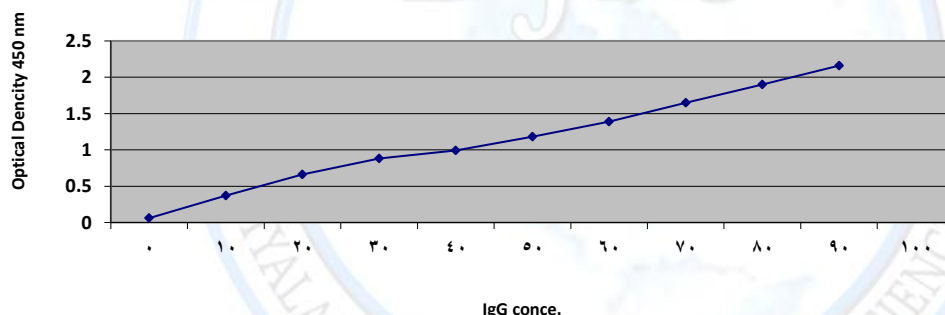


Fig-1 :standard curve to detection of IgG Concentration

Category 1: IgG was taking from [Artificial Immunolab test kit] stander in ordinary ELISA.

Category 2: IgG was Isolated by Ione exchange chromatography technique. [New kit]

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3-Detection of IgG concentration by direct ELISA:**Procedure****1) Coating**

1 -The Antibody type-IgG (IgG was Isolated by Ione Exchange Chromatography Technique) was diluted with coating buffer (Sodium bicarbonate Buffer, pH= 9.6), and appropriate wells of ELISA plate were coated with the IgG by adding (100 μ l) of the diluted purified solution.

Note: The concentration of coated Antibody is 10 μ g/ ml

2. Cover the plate with an adhesive plastic and incubate at 37 °C for 2 hours or at 4 °C overnight.

3. The plate was washed with 200 μ l of washing buffer (PBS) for three times [16].

2) Blocking

4. 200 μ l of blocking buffer (1%) was added (Bovine serum albumin, BSA) to block the non-specific binding sites in the coated wells.

5. The plate was covered with an adhesive plastic and incubates at 37 °C for 1 hour or at 4 °C overnight.

6. Wash the plate with 200 μ l of washing Buffer for three times.

3) Detection

Detection IgG by following steps:

1-Add 100 μ l Enzyme Conjugate: anti-human-IgG-HRP (from-rabbit) on wells were coated by IgG (isolated or standard).2- Cover plate with the enclosed foil and incubate at room temperature for60minutes. 3- Empty the wells of the plate (dump or aspirate) and add 300 μ l of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

4- Pipet 100 each of ready -to-use substrate TMB (Tetramethyl benzidine), into the wells. This time also the substrate blank is pipetted.

5- Cover plate with the enclosed foil and incubate at room temperature for 20 minutes in the dark.

6- Add 50 μ l stop solution (0.5 M sulfuric acid) into the wells and waiting for 5min.

7-After thorough mixing and wiping the bottom of the plate perform the reading of the absorption at 450 nm.

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4- Detection concentration of hydatid antigen in serum by indirect ELISA:**Procedure****1) Coating**

1-Diluted the specific hydatid cyst antigen, which supplied by Human, Lad, Germany with Coating Buffer(sodium bicarbonate buffer (pH 9.6), and coat 6 wells of ELISA plate with the specific hydatid antigen by adding (100 μ l)of the diluted antigen solution(1:100) to made stander curve.

2-Added 100 μ l of serum subject (serum patients before surgery and control, also another ELISA plate used serum patients after surgery), and coat appropriate wells of ELISA plate.

3. Cover the plate with an adhesive plastic and incubate at 37 °C for 2 hours or at 4 °C overnight.

4. Wash the plate with 200 μ l of Washing Buffer(Phosphate buffered saline)(PBS) for three times

2) Blocking

4. Add 200 μ l of Blocking Buffer (1%) (Bovine serum Albumin) BSA to block the non-specific binding sites in the coated wells.

5. Cover the plate with an adhesive plastic and incubate at 37 °C for 1 hour or at 4 °C overnight.

6. Wash the plate with 200 μ l of Washing Buffer for three times.

3) Detection

Detection serum antigen by following steps:

1-Pipet 100 μ l purified IgG (From purified ion exchange chromatography, concentration 10 μ g/ml).

2- Cover plate with the enclosed foil and incubate at room temperature for 60 minutes.

3-Empty the wells of the plate (dump or aspirate) and add 300 μ l of diluted washing solution.

This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

4- Pipet 100 μ l each of ready-to-use conjugate into the wells. Leave one well empty for the substrate blank.

5- Cover plate with the enclosed foil and incubate at room temperature for 30 minutes.

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6- Empty the wells of the plate (dump or aspirate) and add 300 μ l of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

7- Pipet 100 μ l each of the ready-to-use substrate into the wells. This time also the substrate blank is pipetted.

8-Cover plate with the enclosed foil and incubate at room temperature for 20 minutes in the dark.

9 - Pipet 100 μ l each of the ready-to-use stop solution into the wells. Pipet also the substrate blank.

10-After thorough mixing and wiping the bottom of the plate perform the reading of the absorption at 450 nm [16].The value of the optical density OD=5 was the Cut-Off between positive sample and reagent passivity based on the user, all the least of the five values are negative samples while higher values of 5 samples are positive.

Results and discussion:

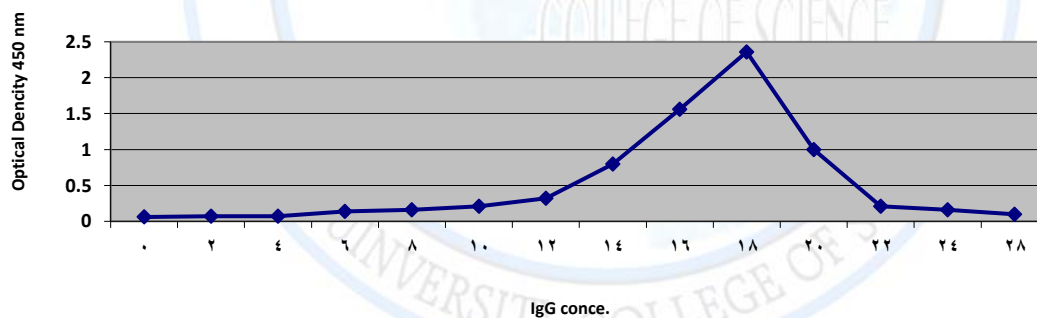


Fig-2 : detection of IgG Concentration

Fig 2/ show that founded IgG high concentration in (12-21) fraction number, this fraction were investigation by ELISA technique (direct principle) clear high concentration of IgG, in this step, isolation and purification of IgG against –hydatid cyst. In this study used ELISA technique to detection antibody that isolation from column chromatography from (12-21) fraction number. These observations were in agreement with other studies [17, 18].

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Table -1: Comparing between standard IgG and isolated IgG

Sample	Type Antibody	The reading of the absorption at 450nm New ELISA Kit
Category 1	specific to CE[Artificial] stander	2.4
Category 2	specific to CE[New]	2.3

Category 1: standard IgG specific to HC.

Category 2: IgG was isolation and purification by chromatography technique. (Table -1) show that standard IgG and isolate IgG (that isolated and purified by chromatography technique) have same OD at 450 nm on direct principle technique , therefore , the new Isolated IgG was may be used as indicator to diagnosis CE infection by ELISA technique.

Table-2: Compare between antigenic concentration ($\mu\text{g/ml}$) in patients pre and post-surgery

Pre-surgery	Post- surgery	Mean of antigen Concentration($\mu\text{g/ml}$)
3.58 \pm 38.71	2.09 \pm 25.70	SE \pm
7.662**		T-test
0.78- 1.63		CI

($P < 0.01$) **CI: Confidant interval

Table -2 and Fig -3 show that increased antigen concentration(before surgery) 38.7 $\mu\text{g/ml}$ than antigen concentration(after surgery) 25.7 $\mu\text{g/ml}$ ($P < 0.01$) due to the circulating hydatid antigens in serum patients body through before period of surgery ,while, the concentration of antigen was decreased after surgical removal of hydatid cyst ,these observations were in agreement with other studies [19,20]. Therefore, some researchers have tried to detect the *E. granulosus* antigens(not antigen concentration) in sera of hydatidosis patients especially after

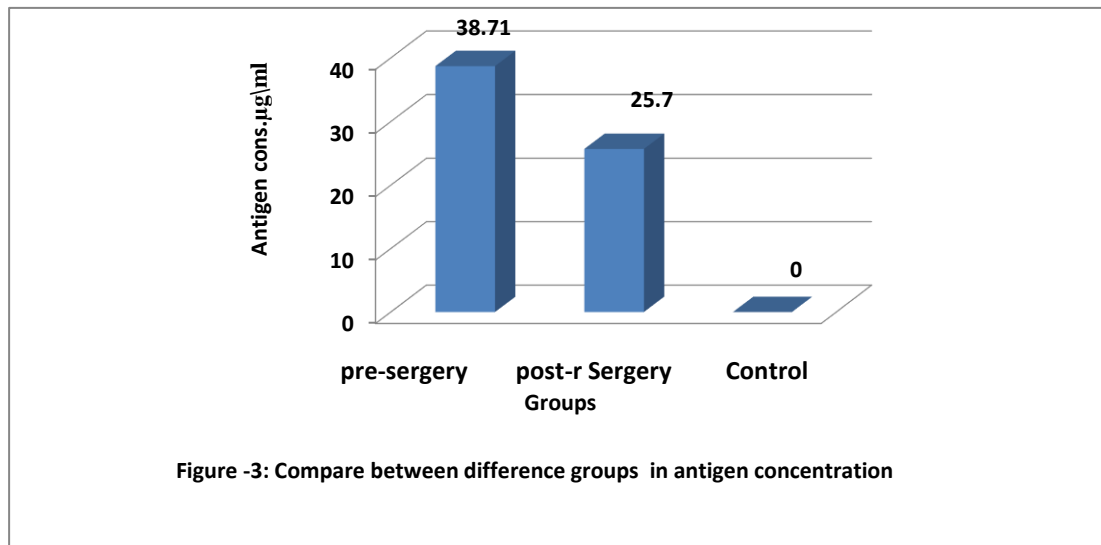
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hydatid cyst surgery , this is the first study that estimates the antigen concentration in the serum of patients before and after surgical removal the hydatid cyst .



The general idea is to detect circulating antigens as well as immune complexes and excreted metacestode antigens in sera by different immunological assays. In a study conducted by Gottstein, [14] hydatid cyst antigen was detected in patients who had not shown anti-hydatid cyst antibodies. Antigen detection assays for diagnosis of hydatidosis resulted in various rates of sensitivity and specificity [7, 14, 9, 12, 19, 20]. In the present study, the presence of circulating antigens in sera of hydatidosis patients by sandwich ELISA (New kit) was detected hydatid cyst antigen in preoperative sera in 50 out of 52 (96.15%), the test demonstrated antigen in 48 out of 52 (92.30%) postoperative sera collected on 3-7 days. These results might be related to the fact that most of antigens in sera are immune complexes of antigens and antibodies which cannot be easily detected by serological assays. Moreover, the intact cysts, small cysts, and cysts in privileged sites do not release enough antigens to be detected in the serum. The antibody is not raised in some of the hydatidosis patients or the titer is low especially in old persons and infants. Also in cerebral, ocular, and calcified cysts, the antibody titer is low and cannot be easily detected Ravinder *et al.*, [21]. On the other hand, the long persistence of anti- *E. granulosus* antibodies after surgical removal of the cysts results in unreliable diagnosis of relapse in patients [22].

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Conclusions

Antigen detection assay might be a useful approach for assessment of the efficacy of treatment especially after removal of the cyst, while antibody detection assay is a sensitive approach for diagnosis of hydatid cyst.

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